PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 September 2000 (12.09.00)	
International application No. PCT/GB00/00146	**Applicant's or agent's file reference** 9.69260/001
International filing date (day/month/year) 20 January 2000 (20.01.00)	**Priority date** (day/month/year) 22 January 1999 (22.01.99)
Applicant RONAGHI, Mostafa	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

16 August 2000 (16.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Copy for the Elected Office (EO/US) PCT/GB00/00146

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

GARDNER, Rebecca
Frank B. Dehn & Co.
179 Queen Victoria Street
London EC4V 4EL
ROYAUME-UNI

Date of mailing (day/month/year)
02 juillet 2001 (02.07.01)

Applicant's or agent's file reference
9.69260/001

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/00146

International filing date (day/month/year)
20 janvier 2000 (20.01.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
GARDNER, Rebeca
Frank B. Dehn & Co.
179 Queen victoria Street
London EC4V 4EL
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Ning Xu
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
FRANK B. DEHN & CO.
Attn. GARDNER, REBECCA
179 Queen Victoria Street
London EC4V 4EL
UNITED KINGDOM

FILE 69260/001
12 MAY 2000
RECEIVED
ANSD

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 09/05/2000
Applicant's or agent's file reference 9.69260/001	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/GB 00/00146	International filing date *(day/month/year)* 20/01/2000
Applicant PYROSEQUENCING AB et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

DATE NOTED
17/00

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Nina Vercio

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the International application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
9.69260/001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 00146	20/01/2000	22/01/1999
Applicant		
PYROSEQUENCING AB et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 2

   [X] as suggested by the applicant.

   [ ] None of the figures.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00146

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAPLEY : "ENHANCING PCR AMPLIFICATION AND SEQUENCING USING DNA-BINDING PROTEINS" MOL.BIOTECHNOLOGY, vol. 2, 1994, pages 295-298, XP000891994 the whole document ---	1-15
Y	KACZOROWSKI ET AL.: "ASSEMBLY OF 18-NUCLEOTIDE PRIMERS BY LIGATION OF THREE HEXAMERS: SEQUENCING OF LARGE GENOMES BY PRIMER WALKING" ANAL.BIOCHEM., vol. 221, XP000457276 the whole document ---	1-15
Y	US 5 547 843 A (STUDIER F WILLIAM ET AL) 20 August 1996 (1996-08-20) the whole document ---	1-15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2000	09/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hagenmaier, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 28440 A (DZIEGLEWSKA HANNA EVA ;PYROSEQUENCING AB (SE); NYREN PAAL (SE)) 2 July 1998 (1998-07-02) cited in the application the whole document	1-15
Y	WO 98 13523 A (DZIEGLEWSKA HANNA EVA ;PYROSEQUENCING AB (SE); UHLEN MATHIAS (SE);) 2 April 1998 (1998-04-02) cited in the application the whole document	1-15
Y	WO 93 23564 A (CEMUBIOTEKNIK AB ;UHLEN MATHIAS (SE); NYREN PAAL (SE)) 25 November 1993 (1993-11-25) cited in the application the whole document	1-15
Y	WO 98 55653 A (NEXSTAR PHARMACEUTICALS INC) 10 December 1998 (1998-12-10) the whole document	1-15
Y	NYREN P ET AL: "DETECTION OF SINGLE-BASE CHANGES USING A BIOLUMINOMETRIC PRIMER EXTENSION ASSAY" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 244, 1 January 1997 (1997-01-01), pages 367-373, XP000199733 ISSN: 0003-2697 the whole document	1-15
Y	RONAGHI ET AL.: "A SEQUENCING METHOD BASED ON REAL-TIME PYROPHOSPHATE" SCIENCE, vol. 281, July 1998 (1998-07), pages 363-365, XP002135869 cited in the application the whole document	1-15
Y	RONAGHI M ET AL: "REAL-TIME DNA SEQUENCING USING DETECTION OF PYROSPHOSPHATE RELEASE" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 242, 1 November 1996 (1996-11-01), pages 84-89, XP002055379 ISSN: 0003-2697 the whole document	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00146

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5547843 A	20-08-1996	NONE	
WO 9828440 A	02-07-1998	AU 5331198 A	17-07-1998
		EP 0946752 A	06-10-1999
WO 9813523 A	02-04-1998	AU 4391897 A	17-04-1998
		EP 0932700 A	04-08-1999
WO 9323564 A	25-11-1993	AU 4068393 A	13-12-1993
WO 9855653 A	10-12-1998	AU 7813698 A	21-12-1998

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9.7.69260/001		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00146	20/01/2000	22/01/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant PYROSEQUENCING AB et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/08/2000	09.03.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer BROCHADO GARGANTA, M Telephone No. +49 89 2399 8935

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00146

I. Basis f the rep rt

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-22 as originally filed

Claims, No.:

1-15 as originally filed

Drawings, sheets:

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-10,12-15
	No:	Claims	11
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-15
Industrial applicability (IA)	Yes:	Claims	1-15
	No:	Claims	

2. Citations and explanations
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   (A) Rapley: Mol. Biotechnology, vol. 2, 1994, 295-298

   (B) WO 98 13523 A

   (C) US 5 547 843

   (D) Ronaghi et al.: Science, vol. 281, July 1998, pages 363-365

2. Novelty

2.1 The subject-matter of claim 11, referring to the use of a single-stranded nucleic acid binding protein (SSB) in a nucleic acid sequencing-by-synthesis method, is not new in the sense of Article 33(2) PCT. This subject-matter is known from document A, which refers to the use of SSB in direct PCR sequencing (see page 298).

3. Inventive step

3.1 Document B discloses a method for identifying a base at a target position in a single-stranded sample DNA sequence, wherein a primer which hybridises to the sample DNA immediately adjacent to the target position is provided and the sample DNA and extension primer are subjected to a polymerase reaction in the presence of a deoxynucleotide, whereby this nucleotide will only become incorporated and release pyrophosphate (PPi) if it is complementary to the base in the target position and said incorporation being detected (see claim 1).

The **only diff renc** between the subject-matter of claim 1 and the disclosure of document B is the fact that a **SSB is included in th polym rase reaction st p**.

The problem to be solved by the present application may therefore be regarded as to provide an **improved method** for identifying a base at a target position in a sample.

Document A discloses the use of SSB for enhancing PCR amplification and sequencing (see title). Although no effect of SSB could be found with regard to the PCR amplification, this situation was different when **using SSB in direct PCR sequencing**, whereby a **dramatic increase in signal intensity has been detect d**. SSB is thought to prevent complementary strand reassociation following the annealing of the sequencing primer and probably may also prevent or reduce the formation of any DNA secondary structure, which is known to cause polymerase pausing and premature termination in sequencing reactions (see page 298).

Documents B and A refer both to direct PCR sequencing, i.e. sequencing by synthesis. Thus, it would be obvious for the skilled person to combine the features set out in both documents and arrive in this way to the method of claim 1. Therefore, claim 1 is not based on an inventive concept as required by Article 33(3) PCT.

Moreover, document C refers to the effect of SSB in promoting the specific alignment of short oligonucleotides on a nucleic acid polymer (see abstract), wherein the addition of SSB in sequencing procedures leads to an improved sequence determination (see column 5). In this case, SSB is used in a different sequencing method, but the skilled person would certainly be capable of using this teaching for improving the method of document B and arrive without the need of an inventive concept to the features of the method of claim 1.

3.2 Claims 2-10 do not contain any additional features which in combination with the features of claim 1, meet the requirements of the PCT in respect of inventive step (Article 33(3) PCT).

The additional features of claims 2 and 3 are disclosed in document A (see respectively, pages 295 and 296). The additional features of claims 4-7 are known from document B (see respectively, claim 1; claim 9; claims 1 and 3; claim 2) and the features of claims 9-10 are described in document C (see columns 3, 5 and 9).

The additional features of claim 8, i.e. the presence of apyrase during the polymerase reaction, are commonly used in sequencing methods based on real-time pyrophosphate. For example, document D describes a sequencing method using apyrase for continuously degradating the added nucleotides (see title and figure 1). This adaptation of the method disclosed in document B is certainly know to the skilled person, specially considering that documents B and D are from the same author.

3.3 Claims 12-14 relate to methods for enhancing the activity of a nucleotide-degradating enzyme when used in a nucleic acid sequencing-by-synthesis method, for enhancing the activity of luciferase when used as a detection enzyme, and for maintaining a constant signal intensity during the method of nucleic acid sequencing-by-synthesis.

With regard to the reasoning given in 3.1 and with further regard to the fact that the objectives of the method of claims 12-14 appear to be implicitly met by the mode of action of SSB, these claims cannot be considered to be based on an inventive concept (Article 33(3) PCT).

3.4 Document B refers to a kit for use in a method of sequencing-by-synthesis, comprising nucleotides, a polymerase and means for detection of the incorporation (see claim 10).

The difference between the subject-matter of claim 15 and the disclosure of document B, is the presence of a SSB.

Using the same reasoning as given in 3.1, the subject-matter of claim 15 cannot be considered to be based on an inventive concept as required by Article 33(3) PCT.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GARDNER, Rebecca
FRANK B. DEHN & CO.
179 Queen Victoria Street
London EC4V 4EL
GRANDE BRETAGNE

FILE 69260/001
15 MAR 2001
ANSD 80

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	09.03.2001
Applicant's or agent's file reference 9.7.69260/001	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00146	20/01/2000	22/01/1999

Applicant
PYROSEQUENCING AB et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Pedersen, C Tel.+49 89 2399 8161

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9.7.69260/001		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00146	20/01/2000	22/01/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant PYROSEQUENCING AB et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/08/2000	09.03.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer BROCHADO GARGANTA, M Telephone No. +49 89 2399 8935

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00146

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-22 as originally filed

Claims, No.:

1-15 as originally filed

Drawings, sheets:

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-10,12-15
	No:	Claims	11
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-15
Industrial applicability (IA)	Yes:	Claims	1-15
	No:	Claims	

2. Citations and explanations
   see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   (A) Rapley: Mol. Biotechnology, vol. 2, 1994, 295-298

   (B) WO 98 13523 A

   (C) US 5 547 843

   (D) Ronaghi et al.: Science, vol. 281, July 1998, pages 363-365

2. Novelty

2.1 The subject-matter of claim 11, referring to the use of a single-stranded nucleic acid binding protein (SSB) in a nucleic acid sequencing-by-synthesis method, is not new in the sense of Article 33(2) PCT. This subject-matter is known from document A, which refers to the use of SSB in direct PCR sequencing (see page 298).

3. Inventive step

3.1 Document B discloses a method for identifying a base at a target position in a single-stranded sample DNA sequence, wherein a primer which hybridises to the sample DNA immediately adjacent to the target position is provided and the sample DNA and extension primer are subjected to a polymerase reaction in the presence of a deoxynucleotide, whereby this nucleotide will only become incorporated and release pyrophosphate (PPi) if it is complementary to the base in the target position and said incorporation being detected (see claim 1).

The **only difference** between the subject-matter of claim 1 and the disclosure of document B is the fact that a **SSB is included in the polymerase reaction step.**

The problem to be solved by the present application may therefore be regarded as to provide an **improved method** for identifying a base at a target position in a sample.

Document A discloses the use of SSB for enhancing PCR amplification and sequencing (see title). Although no effect of SSB could be found with regard to the PCR amplification, this situation was different when **using SSB in direct PCR sequencing**, whereby a **dramatic increase in signal intensity has been detected**. SSB is thought to prevent complementary strand reassociation following the annealing of the sequencing primer and probably may also prevent or reduce the formation of any DNA secondary structure, which is known to cause polymerase pausing and premature termination in sequencing reactions (see page 298).

Documents B and A refer both to direct PCR sequencing, i.e. sequencing by synthesis. Thus, it would be obvious for the skilled person to combine the features set out in both documents and arrive in this way to the method of claim 1. Therefore, claim 1 is not based on an inventive concept as required by Article 33(3) PCT.

Moreover, document C refers to the effect of SSB in promoting the specific alignment of short oligonucleotides on a nucleic acid polymer (see abstract), wherein the addition of SSB in sequencing procedures leads to an improved sequence determination (see column 5). In this case, SSB is used in a different sequencing method, but the skilled person would certainly be capable of using this teaching for improving the method of document B and arrive without the need of an inventive concept to the features of the method of claim 1.

3.2 Claims 2-10 do not contain any additional features which in combination with the features of claim 1, meet the requirements of the PCT in respect of inventive step (Article 33(3) PCT).

The additional features of claims 2 and 3 are disclosed in document A (see respectively, pages 295 and 296). The additional features of claims 4-7 are known from document B (see respectively, claim 1; claim 9; claims 1 and 3; claim 2) and the features of claims 9-10 are described in document C (see columns 3, 5 and 9).

The additional features of claim 8, i.e. the presence of apyrase during the polymerase reaction, are commonly used in sequencing methods based on real-time pyrophosphate. For example, document D describes a sequencing method using apyrase for continuously degradating the added nucleotides (see title and figure 1). This adaptation of the method disclosed in document B is certainly know to the skilled person, specially considering that documents B and D are from the same author.

3.3 Claims 12-14 relate to methods for enhancing the activity of a nucleotide-degradating enzyme when used in a nucleic acid sequencing-by-synthesis method, for enhancing the activity of luciferase when used as a detection enzyme, and for maintaining a constant signal intensity during the method of nucleic acid sequencing-by-synthesis.

With regard to the reasoning given in 3.1 and with further regard to the fact that the objectives of the method of claims 12-14 appear to be implicitly met by the mode of action of SSB, these claims cannot be considered to be based on an inventive concept (Article 33(3) PCT).

3.4 Document B refers to a kit for use in a method of sequencing-by-synthesis, comprising nucleotides, a polymerase and means for detection of the incorporation (see claim 10).

The difference between the subject-matter of claim 15 and the disclosure of document B, is the presence of a SSB.

Using the same reasoning as given in 3.1, the subject-matter of claim 15 cannot be considered to be based on an inventive concept as required by Article 33(3) PCT.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

GARDNER, Rebecca
Frank B. Dehn & Co.
179 Queen Victoria Street
London EC4V 4EL
ROYAUME-UNI

Date of mailing (day/month/year) 12 September 2000 (12.09.00)	
Applicant's or agent's file reference 9.69260/001	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB00/00146	20 January 2000 (20.01.00)	22 January 1999 (22.01.99)

Applicant
PYROSEQUENCING AB et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
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   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
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3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

NAT PHASES Due.
22/7/01.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer: Olivia TEFY
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: PYROSEQUENCING AB [SE/SE]; Vallongatan 1, S–752 28 Uppsala (SE).

(71) Applicant *(for GB only)*: GARDNER, Rebeca [GB/GB]; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: RONAGHI, Mostafa [SE/US]; Stanford DNA Sequencing & Technology Center, 855 California Avenue, Palo Alto, CA 94304 (US).

(74) Agents: GARDNER, Rebecca et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).

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Published
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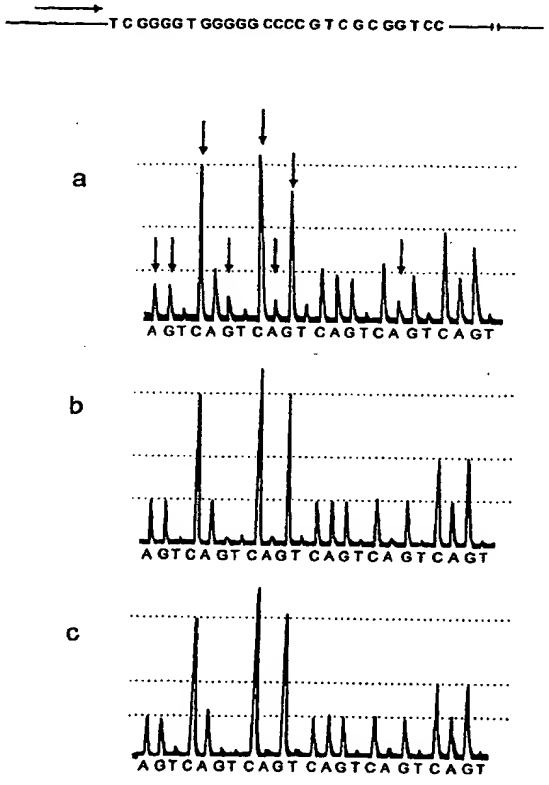
(54) Title: A METHOD OF DNA SEQUENCING

(57) Abstract

The present invention relates to a method of identifying a base at a target position in a sample nucleic acid sequence wherein a primer, which hybridises to the sample nucleic acid immediately adjacent to the target position, is provided and the sample nucleic acid and primer are subjected to a polymerase reaction in the presence of a nucleotide whereby the nucleotide will only become incorporated if it is complementary to the base in the target positon, and said incorporation is detected, characterised in that, a single–stranded nucleic acid binding protein is included in the polymerase reaction step.



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A method of DNA sequencing

The present invention relates to methods of nucleic acid sequencing and in particular to sequencing-by-synthesis methods, ie. those methods based on the detection of nucleotide incorporation during polymerase extension, rather than on analysis of the nucleotide sequence itself, and to the improvements derivable in such methods by the use of a single-stranded DNA binding protein.

DNA sequencing is an essential tool in molecular genetic analysis. The ability to determine DNA nucleotide sequences has become increasingly important as efforts have commenced to determine the sequences of the large genomes of humans and other higher organisms. The two most commonly used methods for DNA sequencing are the enzymatic chain-termination method of Sanger and the chemical cleavage technique of Maxam and Gilbert. Both methods rely on gel electrophoresis to resolve, according to their size, DNA fragments produced from a larger DNA segment. Since the electrophoresis step as well as the subsequent detection of the separated DNA-fragments are cumbersome procedures, a great effort has been made to automate these steps. However, despite the fact that automated electrophoresis units are commercially available, electrophoresis is not well suited for large-scale genome projects or clinical sequencing where relatively cost-effective units with high throughput are needed. Thus, the need for non-electrophoretic methods for sequencing is great and several alternative strategies have been described, such as scanning tunnel electron microscopy (Driscoll et al., 1990, Nature, 346, 294-296), sequencing by hybridization (Bains et al., 1988, J. Theo. Biol. 135, 308-307) and single molecule detection (Jeff et al., 1989, Biomol.

Struct. Dynamics, 7, 301-306), to overcome the disadvantages of electrophoresis.

Techniques enabling the rapid detection of a single DNA base change are also important tools for genetic analysis. In many cases detection of a single base or a few bases would be a great help in genetic analysis since several genetic diseases and certain cancers are related to minor mutations.

Sequencing-by-synthesis methods are useful ways of determining the sequence of a DNA molecule of up to a hundred or more bases or the identity of a single nucleotide within a sample DNA molecule. During typical sequencing-by-synthesis methods the four different nucleotides (adenine, thymine, guanine and cytosine) are conveniently added cyclically in a specific order; when the base which forms a pair (according to the normal rules of base pairing, A-T and C-G) with the next base in the single-strand target sequence is added, it will be incorporated into the growing complementary strand by a polymerase and this incorporation will trigger a detectable signal. The event of incorporation can be detected directly or indirectly. In direct detection, nucleotides are usually fluorescently labelled allowing analysis by a fluorometer. (US Patent 48638449, US Patent 5302509, Metzker et al. Nucl. Acids Res. (1994) 22: 4259-4267, Rosenthal International Patent Application No. WO 93/213401, WO 91/06678, Canard et al. Gene (1994) 148: 1-6). One such strategy of sequencing-by-synthesis called base addition sequencing scheme (BASS) is based on nucleotide analogues that terminate DNA synthesis. BASS involves repetitive cycles of incorporation of each successive nucleotide, *in situ* monitoring to identify the incorporated base, and deprotection to allow the next cycle of DNA synthesis.

Indirect detection usually takes advantage of enzymatic detection, e.g. measuring the release of PPi (inorganic pyrophosphate) during a polymerization

reaction (WO 93/23564 and WO 89/09283). As each nucleotide is added to a growing nucleic acid strand during a polymerase reaction, a pyrophosphate molecule is released. It has been found that pyrophosphate released under these conditions can be detected enzymatically e.g. by the generation of light in the luciferase-luciferin reaction. Such methods enable a base to be identified in a target position and DNA to be sequenced simply and rapidly whilst avoiding the need for electrophoresis and the use of harmful radiolabels. These methods based on release of PPi are referred to herein as Pyrosequencing. The basic PPi-based sequencing methods have been improved by using a dATP analogue in place of dATP (WO 98/13523) and including a nucleotide-degrading enzyme such as apyrase during the polymerase reaction step, so that unincorporated nucleotides are degraded, as described in WO 98/28440.

However, these sequencing-by-synthesis methods mentioned above are not without drawbacks. A particular problem arises when the DNA to be sequenced has a number of identical adjacent bases, especially 3 or more the same. Figure 1 shows the trace obtained when a single-stranded PCR product is sequenced according to known sequencing-by-synthesis methods (in this case involving detection of PPi). Figure 1 shows that known methods do not provide clear results when two or more adjacent bases in the sample molecule are the same. For example, the peak height when the first set of three adenine residues are incorporated is almost the same as when four thymine residues are incorporated later; the set of three adenine residues incorporated around the middle of the sequence have the same peak height as previous doublets and the last pair of adenine residues to be incorporated have a peak height corresponding to single bases from the earlier part of the sequence.

Other problems of sequencing-by-synthesis methods include false signals which are the result of

mispriming, i.e. hybridisation of the primer not to its targeted complement within the target DNA sequence but to another region which will result in generation of "incorporation signals" which do not reflect the identity of the target sequence. There is an associated problem which can result in a false indication of incorporation termed "minus frame incorporation", where a proportion of the growing primer originating strands are not fully extended and false positive signals appear in subsequent cycles.

Thus, there is a need further to improve sequencing-by-synthesis methods by addressing the above problems and more generally to improve the accuracy of the methods while providing methods which are simple and quick to perform, lending themselves readily to automation.

It has surprisingly been found that including a single-stranded nucleic acid binding protein in the reaction mixture improves the ratio of signals generated by one, two, three or more adjacent bases and reduces the number of false signals and generally improves the efficacy and reduces the cost of sequencing-by-synthesis methods.

In one aspect, the present invention thus provides a method of identifying a base at a target position in a sample nucleic acid sequence wherein a primer, which hybridises to the sample nucleic acid immediately adjacent to the target position, is provided and the sample nucleic acid and primer are subjected to a polymerase reaction in the presence of a nucleotide whereby the nucleotide will only become incorporated if it is complementary to the base in the target position, and said incorporation is detected, characterised in that, a single-stranded nucleic acid binding protein is included in the polymerase reaction step.

The nucleic acid to be sequenced may be any nucleotide sequence it is desirable to obtain sequence

information about. Thus, it may be any polynucleotide, or indeed oligonucleotide sequence. The nucleic acid may be DNA or RNA, and may be natural, isolated or synthetic. Thus, the target DNA may be genomic DNA, or cDNA, or a PCR product or other amplicon etc. Alternatively, the target DNA may be synthetic, and genomic DNA, cDNA or a PCR product etc. may be used as primer. The target (sample) nucleic acid may be used in any convenient form, according to techniques known in the art e.g. isolated, cloned, amplified etc., and may be prepared for the sequencing reaction, as desired, according to techniques known in the art.

The DNA may also be single or double-stranded - whilst a single-stranded DNA template has traditionally been used in sequencing reactions, or indeed in any primer-extension reaction, it is possible to use a double-stranded template; strand displacement, or a localised opening-up of the two DNA strands may take place to allow primer hybridisation and polymerase action to occur.

The sample nucleic acid acts as a template for possible polymerase based extension of the primer and thus may conveniently be referred to as "template" or "nucleic acid template".

In the polymerase reaction, any convenient polymerase enzyme may be used according to choice, as will be described in more detail below. In the case of a RNA template, such a polymerase enzyme may be a reverse transcriptase enzyme. The nucleotide may be any nucleotide sutiable for a polymerase chain extension reaction e.g. a deoxynucleotide or a dideoxynucleotide. The nucleotide may optionally be labelled in order to aid or facilitate detection of nucleotide incorporation. One or more nucleotides may be used.

Nucleotide incorporation by the action of the polymerase enzyme may be detected directly or indirectly, and methods for this are well known in the

art. Representative methods are described for example in US-A-4,863,879 of Melamede. As mentioned above, detection of incorporation may be by means of labelled nucleotides, for example fluorescently labelled nucleotides, as is well known in sequencing procedures known in the art. Alternatively, the event of incorporation may be detected by other means e.g. indirectly. Detection of incorporation also includes the detection of absence of incorporation e.g. lack of a signal. Thus, it may be detected whether or not nucleotide incorporation takes place.

The method of the invention thus has utility in a number of different sequencing methods and formats, including mini-sequencing procedures e.g. detection of single base changes (for example, in detecting point mutations, or polymorphisms, or allelic variations etc). The method of the invention may thus be used in a "full" sequencing procedure, ie. the identification of the sequential order of the bases in a stretch of nucleotides, as well in single base detection procedures.

For example, to determine sequence information in a target nucleotide sequence, different deoxynucleotides or dideoxynucleotides may be added either to separate aliquots of sample-primer mixture or successively to the same sample-primer mixture and subjected to the polymerase reaction to indicate which deoxynucleotide or dideoxynucleotide is incorporated.

In order to sequence the target DNA, the procedure may be repeated one or more times i.e. cyclically, as is known in the art. In this way the identity of many bases in the sample nucleic acid may be identified, essentially in the same reaction.

Hence, a sequencing protocol may involve annealing a primer as described above, performing a polymerase-catalysed primer extension step, detecting the presence or absence of incorporation, and repeating the

nucleotide addition and primer extension steps etc. one or more times. As discussed above, nucleotides may be added singly or individually, or in a mixture, successively to the same primer-template mixture, or to separate aliquots of primer-template mixture, or to separate aliquots of primer-template mixture etc. according to choice, and the sequence information it is desired to obtain.

The term "single-stranded nucleic acid binding protein" as used herein is intended to refer to the class of proteins collectively referred to by the term SSB (Ann. Rev. Biochem. [1986] 55 103-136 Chase et al.). SSB has the general property of preferential binding to single-stranded (ss) over double-stranded (ds) nucleic acid molecules. The class includes *E. coli* single-stranded binding protein (Eco SSB), T4 gene 32 protein (T4 gp32), T7 SSB, coliphage N4 SSB, T4 gene 44/62 protein, adenovirus DNA binding protein (AdDBP or AdSSB), calf thymus unwinding protein (UP1) and the like (Coleman et al. CRC Critical Reviews in Biochemistry, (1980) 7(3), 247-289 and p5 SSB from fi-29 DNA (Lindberg et al. J. Biol. Chem. (1989) 264 12700-08) (Nakashima et al. FEBS Lett. (1974) 43 125). Any functionally equivalent or analogous protein, for example derivatives or modifications of the above-mentioned proteins, may also be used. Eco SSB or derivatives thereof are particularly preferred for use in the methods of the present invention.

Thus, modified single-stranded nucleic acid binding proteins derived by isolation of mutants or by manipulation of cloned single-stranded nucleic acid binding proteins which maintain the advantageous properties described herein, are also contemplated for use in the methods of the invention.

The term "dideoxynucleotide" as used herein includes all 2'-deoxynucleotides in which the 3'-hydroxyl group is absent or modified and thus, while

able to be added to the primer in the presence of the polymerase, is unable to enter into a subsequent polymerisation reaction.

As described above, the method of the invention may be performed in a number of ways, and has utility in a variety of sequencing protocols. Viewed more generally, the present invention can thus be seen to provide the use of a single-stranded nucleic acid binding protein in a nucleic acid sequencing-by-synthesis method. In particular, the single-stranded nucleic acid binding protein is used to bind to the nucleic acid template.

What is meant by a DNA sequencing-by-synthesis method is defined above, namely that sequence information is derived by detecting incorporation of a nucleotide in a primer extension reaction. As explained above, such sequencing-by-synthesis protocols, include not only "full" sequencing methods, but also mini-sequencing methods etc., yielding more limited sequence information.

Any sequencing-by-synthesis method, as described above, is suitable for use in the methods of the present invention but methods which rely on monitoring the release of inorganic pyrophosphate (PPi) are particularly preferred. In this case, incorporation of the nucleotide will be measured indirectly by enzymatic detection of released PPi.

PPi can be determined by many different methods and a number of enzymatic methods have been described in the literature (Reeves et al., (1969), Anal. Biochem., 28, 282-287; Guillory et al., (1971), Anal. Biochem., 39, 170-180; Johnson et al., (1968), Anal. Biochem., 15, 273; Cook et al., (1978), Anal. Biochem. 91, 557-565; and Drake et al., (1979), Anal. Biochem. 94, 117-120).

It is preferred to use luciferase and luciferin in combination to identify the release of pyrophosphate since the amount of light generated is substantially proportional to the amount of pyrophosphate released

which, in turn, is directly proportional to the amount of base incorporated. The amount of light can readily be estimated by a suitable light sensitive device such as a luminometer.

Luciferin-luciferase reactions to detect the release of PPi are well known in the art. In particular, a method for continuous monitoring of PPi release based on the enzymes ATP sulphurylase and luciferase has been developed by Nyren and Lundin (Anal. Biochem., 151, 504-509, 1985) and termed ELIDA (Enzymatic Luminometric Inorganic Pyrophosphate Detection Assay). The use of the ELIDA method to detect PPi is preferred according to the present invention. The method may however be modified, for example by the use of a more thermostable luciferase (Kaliyama et al., 1994, Biosci. Biotech. Biochem., 58, 1170-1171). This method is based on the following reactions:

$$PPi + APS \xrightarrow{\text{ATP sulphurylase}} ATP + SO_4^{2-}$$

$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} AMP + PPi + \text{oxyluciferin} + CO_2 + h\nu$$

(APS = adenosine 5'-phosphosulphate)

The preferred detection enzymes involved in the PPi detection reaction are thus ATP sulphurylase and luciferase. Methods of detecting the light emitted are well known in the art.

In order to repeat the method cyclically and thereby sequence the sample nucleic acid and, also to aid separation of a single-stranded sample DNA from its complementary strand, it may be desirable that sample nucleic acid (DNA) is immobilised or provided with means for immobilisation attachment to a solid support.

Moreover, the amount of sample nucleic acid available may be small and it may therefore be desirable to amplify the sample nucleic acid before carrying out the method according to the invention.

The sample DNA may be amplified, for example *in vitro* by PCR, Self Sustained Sequence Replication (3SR), Rolling Circle Amplification or Replication (RCA or RCR), or indeed any other *in vitro* amplification technique, or *in vivo* using a vector and, if desired, *in vitro* and *in vivo* amplification may be used in combination. Whichever method of amplification is used, it may be convenient to adapt the method such that the amplified nucleic acid becomes immobilised or is provided with means for attachment to a solid support. For example, a PCR primer may be immobilised or be provided with means for attachment to a solid support. Also, a vector may comprise means for attachment to a solid support adjacent the site of insertion of the sample DNA such that the amplified sample DNA and the means for attachment may be excised together.

Immobilisation of the amplified DNA may take place as part of the amplification itself, e.g. in PCR where one or more primers are attached to a support, or alternatively one or more of the primers may carry means for immobilisation e.g. a functional group permitting subsequent immobilisation, e.g. a biotin or thiol group. Immobilisation by the 5' end of a primer allows the strand of DNA emanating from that primer to be attached to a solid support and have its 3' end remote from the support and available for subsequent hybridisation with the extension primer and chain extension by polymerase.

The solid support may conveniently take the form of microtitre wells, or dipsticks which may be made of polystyrene activated to bind the primer DNA (K Almer, Doctoral Theses, Royal Institute of Technology, Stockholm, Sweden, 1988). However, any solid support may conveniently be used, including any of the vast

number described in the art, e.g. for separation/ immobilisation reactions or solid phase assays. Thus, the support may also comprise particles, fibres or capillaries made, for example, of any polymer, e.g. agarose, cellulose, alginate, Teflon or polystyrene. Glass solid supports can also be used, e.g. glass plates or capillaries. Magnetic particles e.g. the superparamagnetic beads produced by Dynal AS (Oslo, Norway) are a preferred support since they can be readily isolated from a reaction mixture yet have superior reaction kinetics over many other forms of support.

The solid support may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups, or other moieties such as avidin or streptavidin, for the attachment of primers or the target nucleic acid. These may in general be provided by treating the support to provide a surface coating of a polymer carrying one of such functional groups, e.g. polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups or an aminoalkylated polymer to provide amino groups. Sulphur and epoxy-based functional groups may also be used. US Patent No. 4654267 describes the introduction of many such surface coatings.

The assay technique is very simple and rapid, thus making it easy to automate by using a robot apparatus where a large number of samples may be rapidly analysed. Since the preferred detection and quantification is based on a luminometric reaction this can be easily followed spectrophotometrically. The use of luminometers is well known in the art and described in the literature.

As mentioned above, the sample nucleic acid, ie. the target nucleic acid to be sequenced, may be any nucleotide sequence, however obtained according to

techniques known in the art, e.g. cloning, DNA isolation etc. It may for example be cDNA synthesised from RNA in the sample and the method of the invention is thus applicable to diagnosis on the basis of characteristic RNA. Such preliminary synthesis can be carried out by a preliminary treatment with a reverse transcriptase, conveniently in the same system of buffers and bases of subsequent amplification e.g. PCR steps, if used. Since the PCR procedure requires heating to effect strand separation, in the case of PCR the reverse transcriptase will be inactivated in the first PCR cycle. When mRNA is the sample nucleic acid, it may be advantageous to submit the initial sample, e.g. a serum sample, to treatment with an immobilised polydT oligonucleotide in order to retrieve all mRNA via the terminal polyA sequences thereof. Alternatively, a specific oligonucleotide sequence may be used to retrieve the RNA via a specific RNA sequence. The oligonucleotide can then serve as a primer for cDNA synthesis, as described in WO 89/0982.

Advantageously, the primer for the polymerase chain extension step (ie. the extension primer) is sufficiently large to provide appropriate hybridisation with the sequence immediately 5' of the target position, yet still reasonably short in order to avoid unnecessary chemical synthesis. It will be clear to persons skilled in the art that the size of the extension primer and the stability of hybridisation will be dependent to some degree on the ratio of A-T to C-G base pairings, since more hydrogen bonding is available in a C-G pairing. Also, the skilled person will consider the degree of homology between the extension primer to other parts of the amplified sequence and choose the degree of stringency accordingly. Guidance for such routine experimentation can be found in the literature, for example, Molecular Cloning: a laboratory manual by Sambrook, J., Fritsch E.F. and Maniatis, T. (1989).

The primer is conveniently added before the sample is divided into (four) aliquots although it may be added separately to each aliquot. It should be noted that the extension primer may be identical with the PCR primer but advantageously it may be different, to introduce a further element of specificity into the system.

Where appropriate, the polymerase reaction is carried out using a polymerase which will incorporate deoxynucleotides and dideoxynucleotides, e.g. T7 polymerase, Klenow, ϕ29 DNA polymerase or Sequenase Ver. 2.0 (USB U.S.A.). Any suitable polymerase may be used and many are known in the art and reported in the literature. However, it is known that many polymerases have a proof-reading or error checking ability and that 3' ends available for chain extension are sometimes digested by one or more nucleotides. If such digestion occurs in the method according to the invention the level of background noise increases. In order to avoid this potential problem, a nonproof-reading polymerase, e.g. T7 polymerase or Sequenase may be used. Otherwise it is desirable to add to each aliquot fluoride ions or nucleotide monophosphates which suppress 3' digestion by polymerase.

A fuller description of preferred embodiments of PPi based sequencing-by-synthesis methods are provided in WO 98/13523 and WO 98/28440 which are incorporated herein by reference. The use of a dATP analogue such as dATPαS in place of dATP is advantageous as it does not interfere with the detection reaction as it is capable of acting as a substrate for a polymerase but incapable of acting as a substrate for the PPi-detection enzyme luciferase. It is therefore possible to perform the chain extension and detection, or signal-generation, reactions substantially simultaneously by including the "detection enzymes" in the chain extension reaction mixture and therefore the sequencing reactions can be continuously monitored in real-time, with a signal being

generated and detected, as each nucleotide is incorporated.

Inclusion of a nucleotide degrading enzyme in the reaction mix is also advantageous as it means that it is not necessary to wash the template thoroughly between each nucleotide addition to remove all non-incorporated deoxynucleotides, which has the associated benefit that a template can be sequenced which is not bound to a solid support.

In a particularly preferred method of the invention, the nucleotide-degrading enzyme apyrase is included during the polymerase reaction step and it has been found that the single-stranded binding proteins used in the methods of the present invention are able to stimulate the activity of apyrase. Whilst not wishing to be bound by theory, it is believed that the "SSB" may play a role in reducing the inhibition of apyrase which may be observed in the presence of DNA.

Thus in a further aspect, the present invention provides a method of enhancing the activity of a nucleotide-degrading enzyme when used in a nucleic acid sequencing-by-synthesis method, which comprises the use of a single-stranded nucleic acid binding protein. More particularly, the methods comprise including or adding a single-stranded nucleic acid binding protein to the sequencing reaction mixture (i.e. the template, primer, polymerase and/or nucleotide (e.g.dNTP/ddNTP) mix).

A similar enhancing effect has been observed on the luciferase enzyme which may be used in signal detection and therefore in a further aspect the present invention provides a method of enhancing the activity of luciferase when used as a detection enzyme in a nucleic acid sequencing-by-synthesis method which comprises the use of a single-stranded nucleic acid binding protein. Again, such methods involve including or adding a single-stranded nucleic acid binding protein to the sequencing reaction mixture.

In a preferred embodiment of the present invention the single-stranded nucleic acid binding protein is added after hybridisation of the primer to the template nucleic acid molecule.

It is also preferred, not to remove the single-stranded nucleic acid binding protein after it has been added.

It is a particular advantage of the present sequencing methods that there need be no separation of the different reagents and enzymes involved in the extension and detection reactions but the labelled or unlabelled nucleotides or nucleotide analogues, sample, polymerase and where appropriate enzymes and enzyme substrates, as well as the single-stranded nucleic acid binding protein can be included in the reaction mixture and there is no need to remove the single-stranded nucleic acid binding protein for detection to take place.

The reaction mixture for the polymerase chain extension step may optionally include additional ingredients or components if desired. Thus, such additional components can include other substances or molecules which bind to DNA. Thus, amines such as spermidine may be used. It was observed that improved results may be obtained using spermidine in run-off extension reactions.

Alternative additional components include DNA binding proteins such as RecA. In particular, it has been observed that a synergy occurs between RecA and a single-stranded nucleic acid binding protein, leading to improved results. Accordingly, the combination of RecA with a single-stranded nucleic acid binding protein represents a preferred embodiment according to the present invention. Other DNA binding proteins involved in DNA replication, recombination, or structural organisation may also be used in similar manner.

Other components which may be included,

particularly when a double stranded substrate is used, include DMSO and formamide, and other agents which may destabilise or assist in destabilising double-strand formation, for example accessory proteins involved in DNA replication such as helicase.

When a single strand nucleic acid binding protein is used in accordance with the methods of the present invention, the methods are robust and results are readily reproducible. The read-length, i.e. the length of nucleic acid which can be successfully sequenced, has been increased by four times as compared to previous sequencing-by-synthesis methods. The methods of the invention are suitable for midi-sequencing, ie. the sequencing of nucleic acid molecules having 9-50 bases, mini-sequencing, the detection of single bases such as SNPs (single nucleotide polymorphisms) responsible for genetic diseases and the sequencing of nucleic acid molecules of 100 bases or more. It is in the successful sequencing of larger molecules that the benefits of a single strand nucleic acid binding protein in, particularly PPi based, sequencing methods are observed. The problems of maintaining a constant signal intensity for incorporation of one nucleotide, two nucleotides and so on, throughout the whole sequencing run are overcome.

In particular, the present invention may advantageously be used in the sequencing of 25 or more, advantageously 30 or more, 50 or more, or 60 or more bases.

As well as increasing the read length, the use of a single stranded nucleic acid binding protein enables the use of longer template molecules. Thus, sequence information from e.g. a 50 base region within a template molecule of 400 or more bases can be obtained. Template molecules of 800 or more, even 1200 or 1500 or more bases can be used in the methods of the present invention.

The amount of sequencing template accessible for

the sequencing reaction may be reduced due to specific and/or unspecific interactions between the template and components of the reaction mixture and/or the surface of the vessel holding the reaction mixture. Such interaction may result in the reduction of the detected signal and/or generation of unspecific sequencing signal. SSB is believed to protect the template from such undesirable interactions and thus improve signal intensity and specificity. Furthermore, the protein may assist in "opening-up" and/or maintaining or stabilising the "open" structure of a double-stranded template.

When using the sequencing methods of the invention to detect mutations in a nucleic acid molecule, the sequencing reaction may advantageously be run bidirectionally to confirm the mutation.

Moreover, a further beneficial feature of the present invention is the stimulation of Klenow polymerase which is observable using the single-stranded nucleic acid binding protein according to the methods described herein.

Thus in a further aspect, the present invention provides a method of maintaining a constant signal intensity during a method of nucleic acid sequencing-by-synthesis comprising the use of a single-stranded nucleic acid binding protein. More particularly, the methods comprise including or adding a single-stranded nucleic acid binding protein to the sequencing reaction mixture (i.e. the template, primer, polymerase and/or nucleotide (e.g.dNTP/ddNTP) mix).

It is to be understood that 'constant signal intensity' in this context means that the strength of the signal, however measured, which indicates incorporation of the correct base-pair nucleotide remains substantially the same throughout the sequencing reaction, whether it is the first nucleotide incorporated, the twentieth or the sixtieth etc. Similarly, the strength of signal indicating

incorporation of two nucleotides (ie. two adjacent bases are the same in the molecule to be sequenced) remains constant throughout the whole sequencing reaction and so on for three bases, four bases etc.

The single-stranded nucleic acid binding protein is present in the 'reaction mixture', i.e. together with the reagents, enzymes, buffers, primer, sample etc. which may include a solid support and is the site of polymerisation and also, where appropriate, detection.

A further benefit of the use of a single-stranded nucleic acid binding protein in accordance with the present invention is the relatively small amount of sample nucleic acid which is required for generation of useful sequence information. Approximately 0.05 pmol DNA in a 50 μl reaction is sufficient to obtain sequence information which means the quantity of enzymes needed for carrying out the extension reactions is less per cycle of nucleotide additions and per full sequencing reaction.

In a further aspect, the present invention provides a kit for use in a method of sequencing-by-synthesis which comprises nucleotides for incorporation, a polymerase, means (e.g. any reagents and enzymes needed) for detection of incorporation and a single-stranded nucleic acid binding protein.

The invention will now be described by way of non-limiting Examples with reference to the Figures in which:

Figure 1 shows a sequencing method of the prior art performed on a 130-base-long single-stranded PCR product hybridized to the sequencing primer. About 2 pmol of the template/primer was used in the assay. The reaction was started by the addition of 0.6 nmol of the indicated deoxynucleotide and the PPi released was detected. The DNA-sequence after the primer is indicated in the Figure.

Figure 2 shows Pyrosequencing of a PCR product a)

in the absence of a single-stranded DNA binding protein, b) in the presence of SSB from T4 phage (T4gp32), and c) in the presence of SSB from *E. coli*. Lower quality of sequence data is obtained in the absence of SSB as indicated by arrows.

Figure 3 shows a) the sequencing of mutated p53 template in the absence of a single strand DNA binding protein and b) sequencing of the same sequence when SSB is included.

Figure 4 shows a run off extension signal obtained on a 1500bp long PCR fragment using Klenow DNA polymerase. A) in the presence of SSB and B) in the absence of a single-stranded DNA binding protein.

Figure 5 shows the result of Pyrosequencing an 800bp long PCR template a) in the presence of SSB and b) in the absence of a single-stranded DNA binding protein.

Figure 6 shows, schematically, inhibition of apyrase in the presence of DNA and the ability of SSB to reduce the interaction of apyrase with the DNA.

Figure 7 shows the Pyrosequencing of a 450bp cDNA template A) in the absence of a single strand DNA binding protein and B) in the presence of SSB. The numbers underneath the peaks show the number of bases incorporated. In A) the dashed line indicates how the strength of signal which results from the incorporation of one base decreases as the sequencing reaction progresses. In B) the dashed lines indicate how the strength of signal as one or two bases are incorporated remains constant when the reaction is carried out in the presence of SSB.

EXAMPLE 1

Comparison of sequencing using *E. coli* SSB, SSB from T4 phage (T4gp32) and no SSB:

Pyrosequencing was performed on approximately 0.2 pmol of a 405-base-long single-stranded PCR product obtained

from mitochondrial DNA hybridised to 2 pmol of sequencing primer pH3A (5'-GCTGTACTTGCTTGTAAGC). Primed DNA template together with 2 μg of SSB from *E. coli* (Amersham Pharmacia Biotech, Uppsala, Sweden) or 2 μg of T4gp32 (Amersham Pharmacia Biotech) was added to a four-enzyme-mixture comprising 6 U DNA polymerase (exonuclease-deficient Klenow DNA polymerase), 20 mU ATP sulfurylase, 200 ng firefly luciferase, and 50 mU apyrase.

The sequencing procedure was carried out by stepwise elongation of the primer-strand upon sequential addition of the different deoxynucleoside triphosphates (Pharmacia Biotech). The reaction was carried out at room temperature. Light was detected by a Pyrosequencer (Pyrosequencing AB, Uppsala, Sweden) as described by Ronaghi et al. (Science 1998, 281: 363-365). See Fig. 2. Unincorporated nucleotides were degraded by apyrase allowing sequential addition of the four different nucleotides in an iterative manner. For this Example the correct sequence is:
5'-AGCCCCACCCCCGGGGCAGCGCCAGG.

EXAMPLE 2

Detection of Mutations:

For mutation detection by Pyrosequencing, 2 pmol of primer COMP53 (5'-GCTATCTGAGCAGCGCTCA) was hybridised to the immobilised single-stranded PCR products obtained from exon 5 of p53 gene from normal and tumour tissues. 1/10 of the template obtained from a PCR product was used in a Pyrosequencing reaction and released light detected as described by Ronaghi et al.(Science 1998, 281: 363-365). There are two altered bases in the mutated template whch can be seen by a signal corresponding to 1.5 bases for T, 0.5 bases for A and 1 base for G. See Fig. 3. The sequence for this Example is 5'-CAT(TA/GG)TGGGGG.

EXAMPLE 3

Stimulation by SSB of Klenow polymerase activity.

A run off extension signal was obtained on a 1500 bp long PCR fragment using Klenow DNA polymerase in the presence of SSB and in the absence of SSB. PCR was performed on 16S rNA gene with ENV1 (U1 universal primer *E. coli* positions 8-27) 5'-AGAGTTTGATIITGGCTCAG and ENV2B (U8 universal primer *E. coli* positions 1515-1493) 5'-B-CGGITACCTTGTTACGACTT. After alkali treatment, the obtained single-stranded template (1/20 of a PCR product) was hybridised to 0.5 pmol of ENV1 primer for a run off extension reaction. 0.5 μg of *E. coli* SSB was added before extension using a coupled enzymatic reaction as described by Nyrén et al. (Anal. Biochem. 1997 244, 367-373). See Fig. 4.

EXAMPLE 4

Pyrosequencing of an 800 bp long PCR template in the presence of SSB, and in the absence of SSB.

Pyrosequencing was performed on an 800-base-long single-stranded PCR product hybridised to the sequencing primer FSS-SEQ-DOWN(5'-CTGCTCGGGCCCAGATCTG). Two μg of SSB from *E. coli* was added to the template/primer (1/5 of a PCR product in which 5 pmol of primers have been used for *in vitro* amplification) and the obtained complex was used in a Pyrosequencing reaction as described by Ronaghi et al. (Science 1998, 281: 363-365). The sequence obtained by Pyrosequencing using SSB is as follows: ATACCGGTCCGGAATTCCCGGTCGACCCACGCGCCGGGCCATCGCA CTTCGCCCACGTGTCGTTTTC). See Fig. 5.

EXAMPLE 5

Inhibition of apyrase by SSB in presence of DNA

To a Pyrosequencing mixture containing 200 ng luciferase

and 50 mU apyrase, is added 4 pmol of ATP, and a signal is obtained. When the degradation curve reaches the base-line, 20 pmol of Romo70A (a 70-base-long oligonucleotide) is added to the solution (nucleotide degradation is inhibited 2.2 times by apyrase shown by a longer time needed to level off to the base line). When 2 μg of SSB is added to the solution, the interaction of the apyrase with the DNA is diminished and apyrase freely functions in the solution and a similar signal as before oligonucleotide addition is obtained. Further addition of 4 pmol ATP shows approximately the same degradation rate as before oligonucleotide addition to the solution. SSB is thus effectively able to stimulate apyrase activity. See Fig. 6.

Example 6

Role of SSB in maintaining a constant signal during the sequencing reaction

Pyrosequencing of a 450 base-long cDNA template obtained by PCR using universal primers was performed in the absence of SSB, and in the presence of SSB. The 450bp single-stranded PCR product was hybridized to the sequencing primer FSS-SEQ-DOWN(5'-CTGCTCGGGCCCAGATCTG). 2 μg of SSB from *E. coli* was added to the template/primer (1/5 of a PCR product in which 5 pmol of primers have been used for in vitro amplification) and the obtained complex was used in a Pyrosequencing reaction as described by Ronaghi et al. (Science 1998, 281:363-365). The sequence obtained by Pyrosequencing using SSB is: ATACCGGTCCGGAATTCCCGGGTCGACCCACGCA
See Fig. 7.

Claims

1. A method of identifying a base at a target position in a sample nucleic acid sequence wherein a primer, which hybridises to the sample nucleic acid immediately adjacent to the target position, is provided and the sample nucleic acid and primer are subjected to a polymerase reaction in the presence of a nucleotide whereby the nucleotide will only become incorporated if it is complementary to the base in the target position, and said incorporation is detected, characterised in that, a single-stranded nucleic acid binding protein is included in the polymerase reaction step.

2. A method as claimed in claim 1 wherein the single-stranded nucleic acid binding protein is selected from the group comprising *E. coli* single-stranded binding protein (Eco SSB), T4 gene 32 protein (T4 gp32), T7 SSB, coliophage N4 SSB, T4 gene 44/62 protein, adenovirus DNA binding protein (AdDBP or AdSSB) and calf thymus unwinding protein (UP1).

3. A method as claimed in claim 2 wherein the single-stranded nucleic acid binding protein is Eco SSB.

4. A method as claimed in any of the preceding claims wherein the sample nucleic acid is DNA.

5. A method as claimed in any of the preceding claims wherein the single-stranded nucleic acid binding protein binds to the sample nucleic acid.

6. A method as claimed in any of the preceding claims wherein the incorporation of the nucleotide is detected by monitoring the release of inorganic pyrophosphate.

7. A method as claimed in claim 6 wherein the release of inorganic pyrophosphate is detected using ATP sulphurylase and luciferase.

8. A method as claimed in any of the preceding claims wherein apyrase is present during the polymerase reaction.

9. A method as claimed in any one of the preceding claims wherein the single-stranded nucleic acid binding protein is added after hybridisation of the primer to the sample nucleic acid.

10. A method as claimed in any one of the preceding claims wherein at least 25 bases in the nucleic acid sample are identified.

11. Use of a single-stranded nucleic acid binding protein in a nucleic acid sequencing-by-synthesis method.

12. A method of enhancing the activity of a nucleotide-degrading enzyme when used in a nucleic acid sequencing-by-synthesis method, which comprises the use of a single-stranded nucleic acid binding protein.

13. A method of enhancing the activity of luciferase when used as a detection enzyme in a nucleic acid sequencing-by-synthesis method which comprises the use of a single-stranded nucleic acid binding protein.

14. A method of maintaining a constant signal intensity during a method of nucleic acid sequencing-by-synthesis comprising the use of a single-stranded nucleic acid binding protein.

15. A kit for use in a method of sequencing-by-synthesis which comprises nucleotides for incorporation, a polymerase, means for detection of incorporation and a single-stranded nucleic acid binding protein.

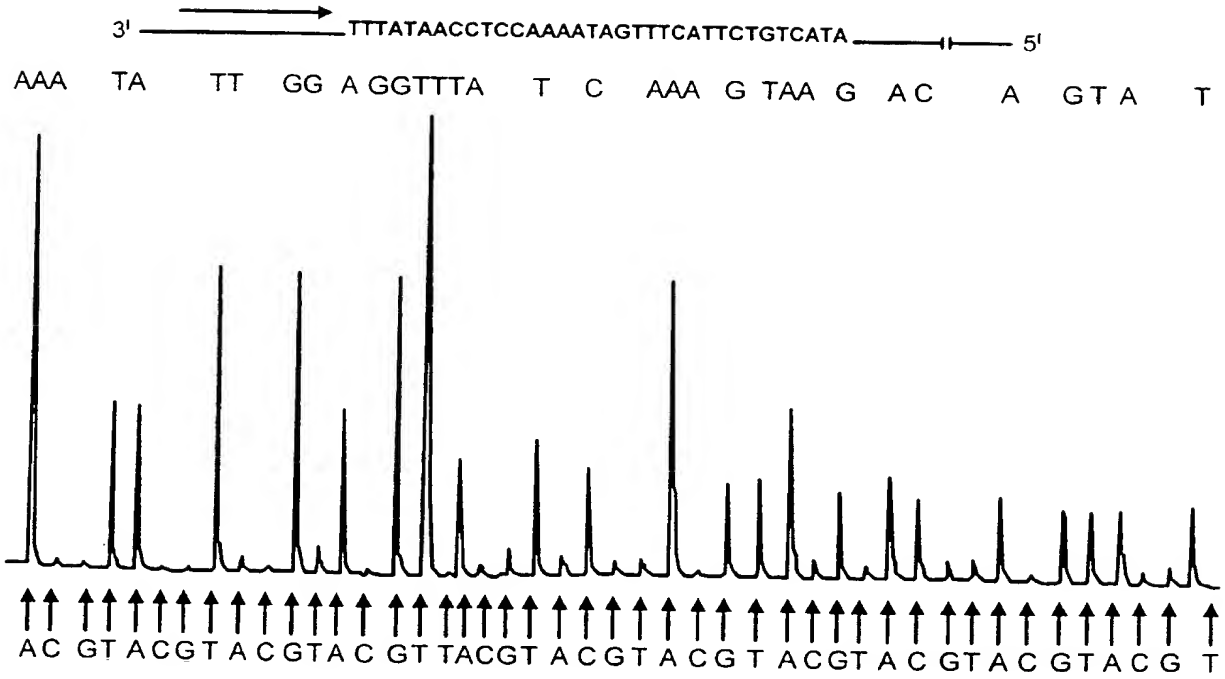
3'
TTTATAACCTCCAAAATAGTTTCATTCTGTCATA
5'
AAA TA TT GG A GGTTTA T C AAA G TAA G AC A GT A T
AC GT ACGT A CGTA C GT TACGT A CGT A CG T ACGT A C GTA C GTA CG T

FIG. 1


T C GGGG T GGGGG CCCC G T C G C GG T CC

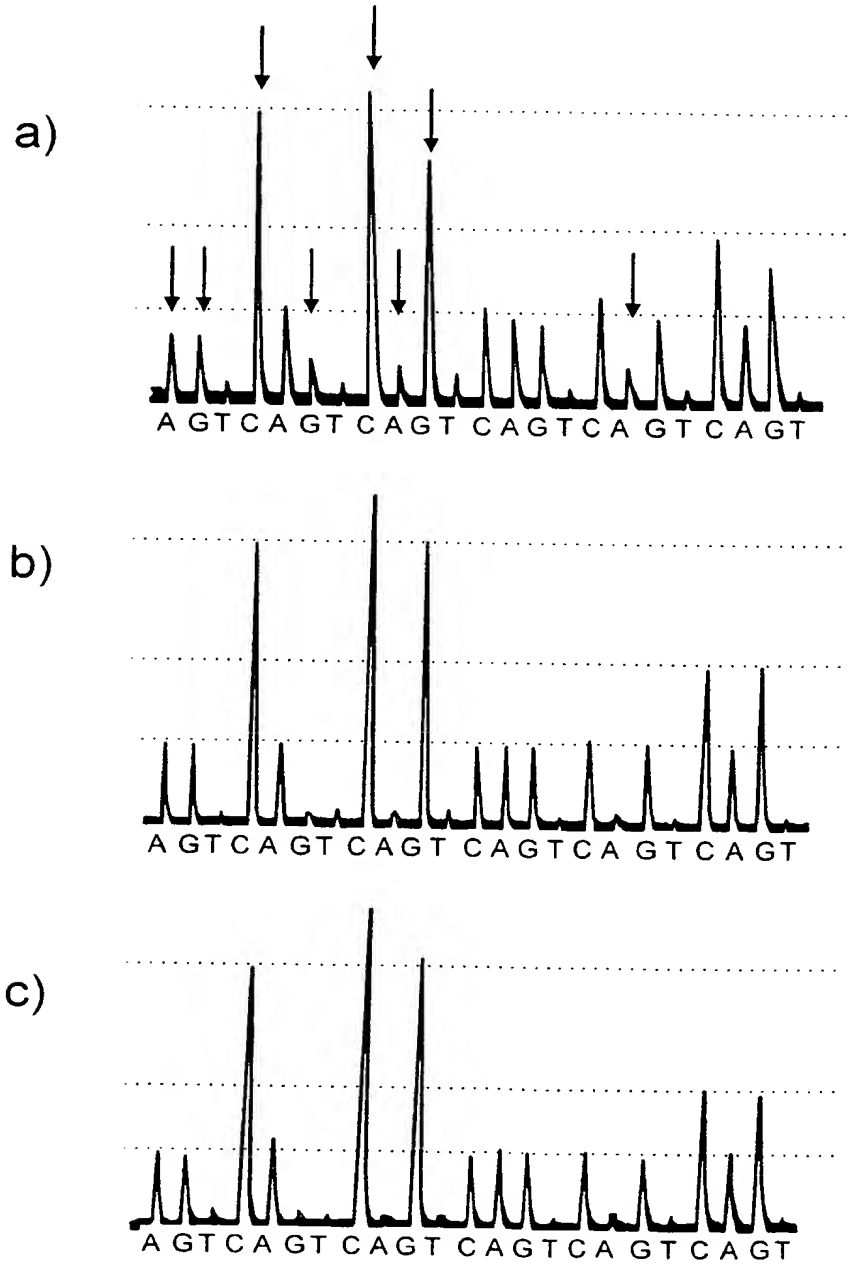
a)
A GT CA GT CA GT CA GT CA GT CA GT
b)
A GT CA GT CA GT CA GT CA GT CA GT
c)
A GT CA GT CA GT CA GT CA GT CA GT

FIG. 2

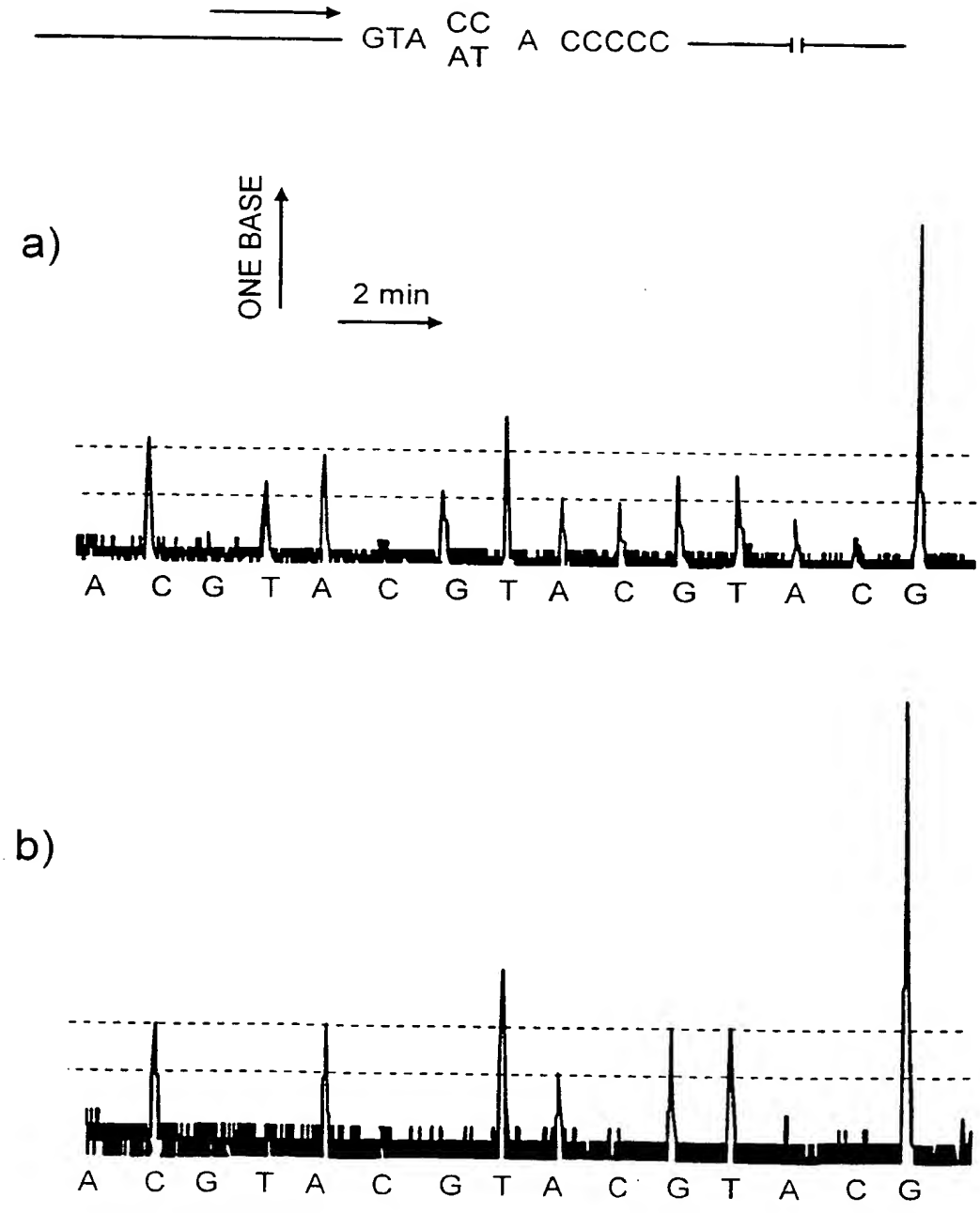
GTA CC AT A CCCCC
a)
ONE BASE
2 min
A C G T A C G T A C G T A C G
b)
A C G T A C G T A C G T A C G

FIG. 3

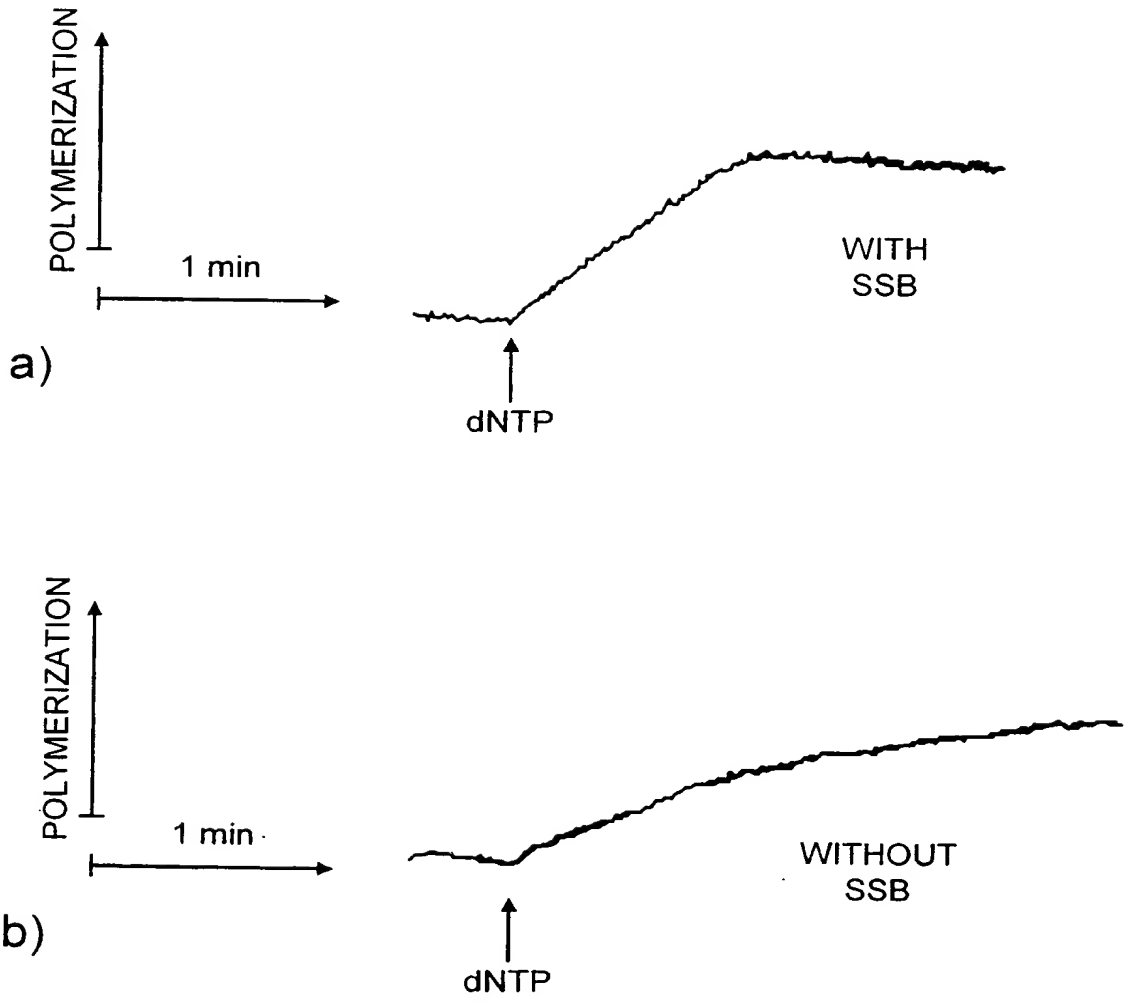
POLYMERIZATION
1 min
WITH
SSB
a)
dNTP
POLYMERIZATION
1 min
WITHOUT
SSB
b)
dNTP

FIG. 4

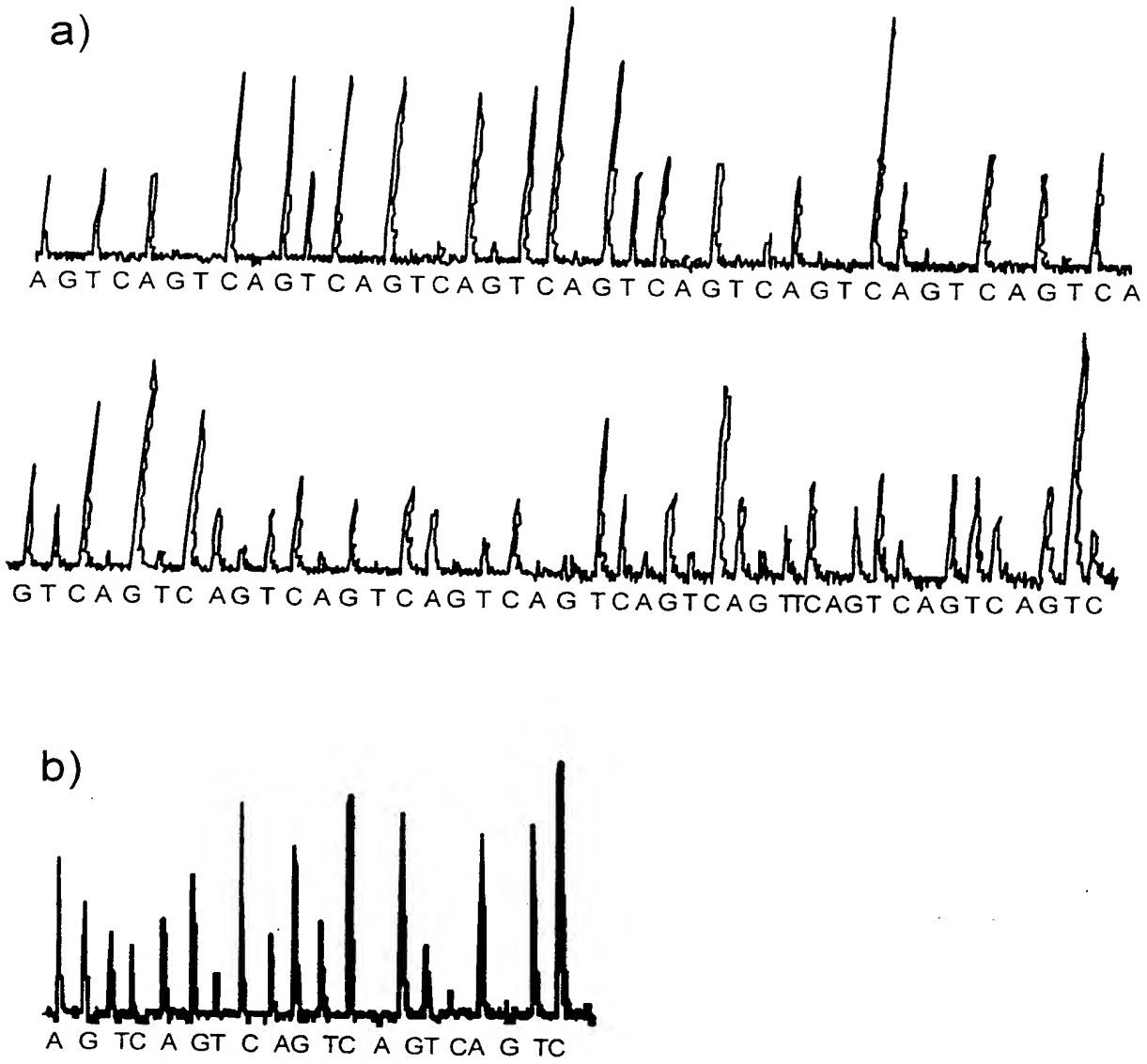
a)
A G T C A G T C A G T C A G T C A G T C A G T C A G T C A G T C A G T C A G T C A
G T C A G T C AG T C A G T C A G T C A G T C A G T C A G T C A G TT C A G T C A G T C AG T C
b)
A G TC A GT C AG TC A GT CA G TC

FIG. 5

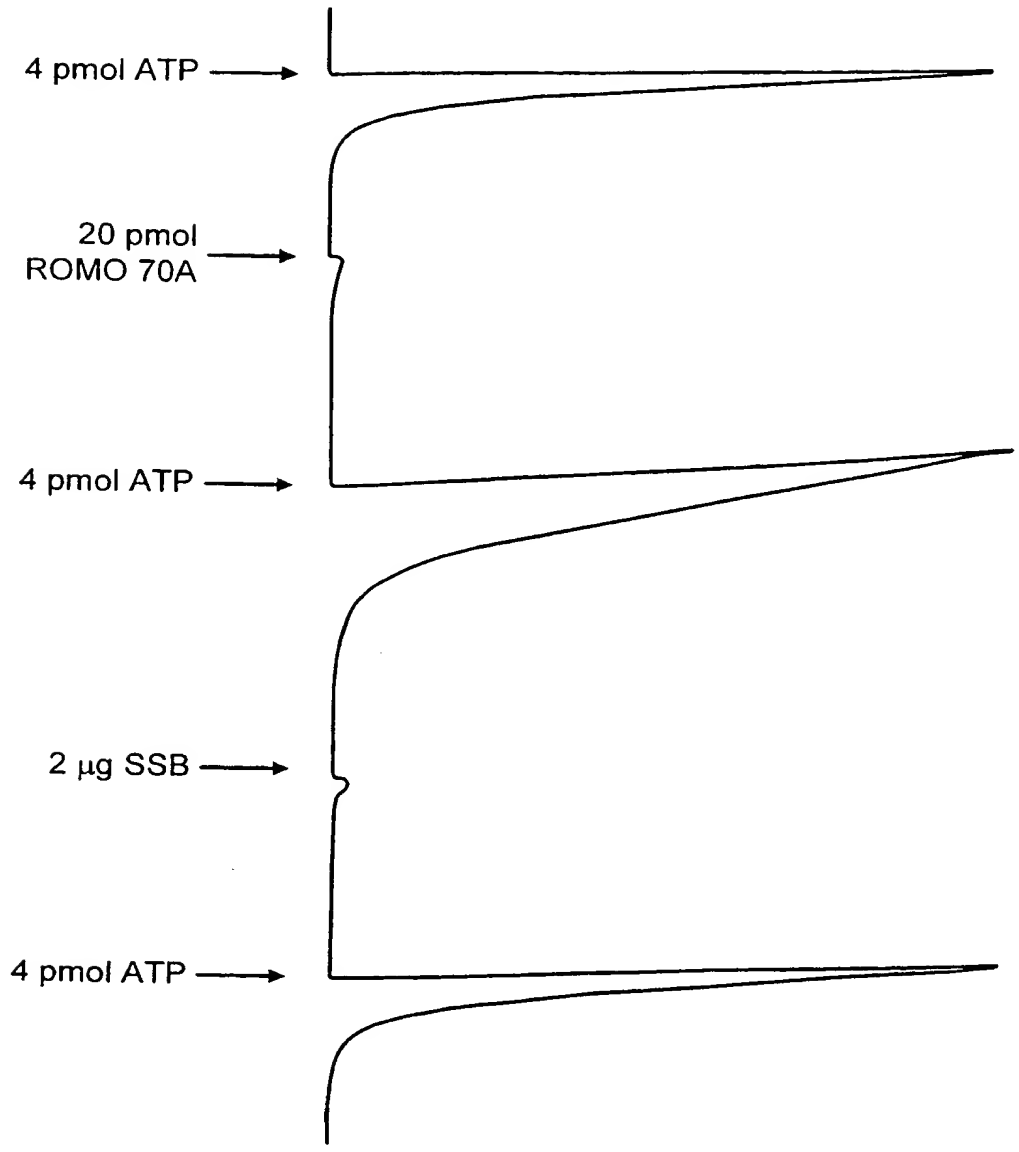

FIG. 6

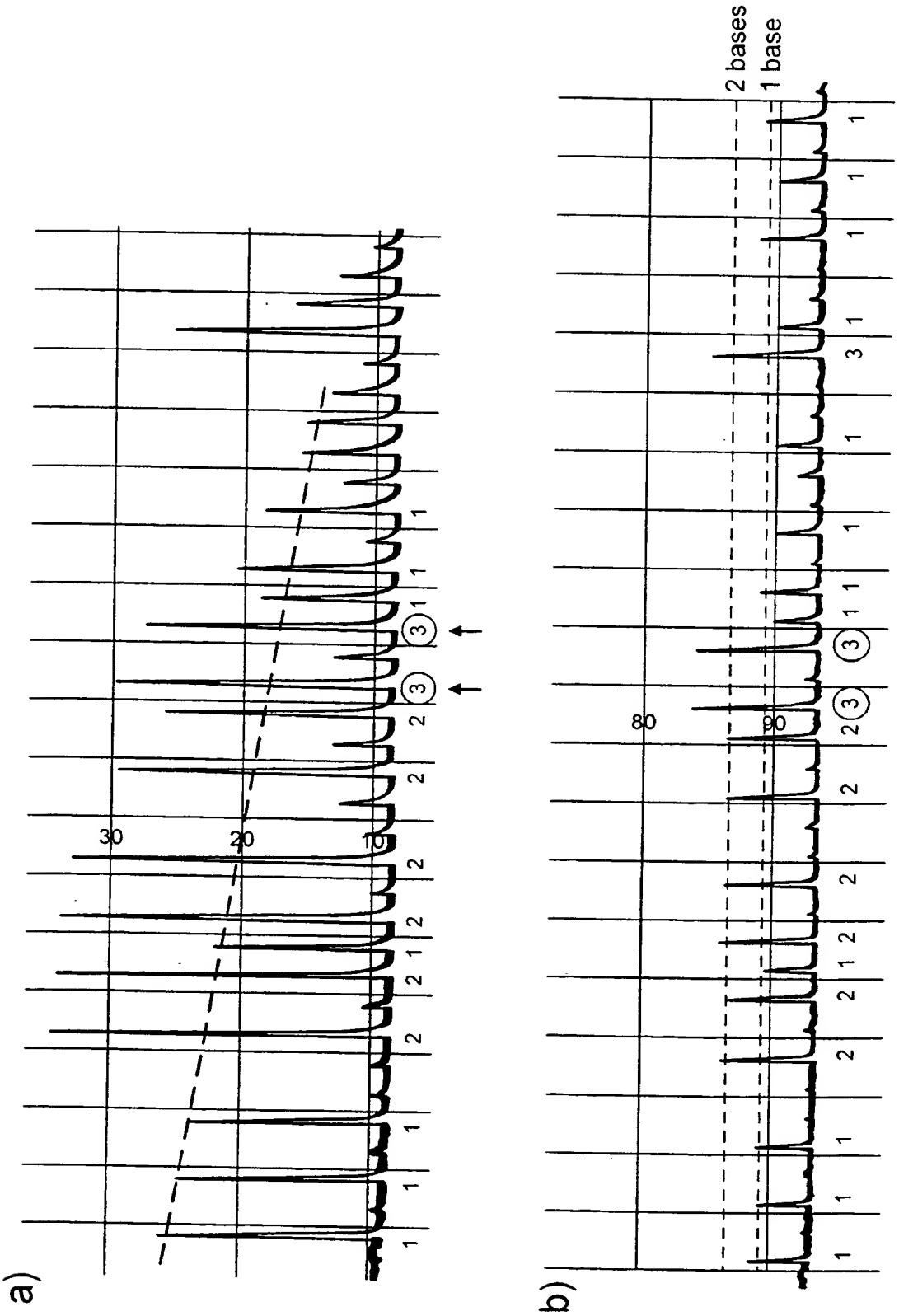

FIG. 7